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# Effects of the glutamate carboxypeptidase II (*GCP2* 1561C>T) and reduced folate carrier (*RFC1* 80G>A) allelic variants on folate and total homocysteine levels in kidney transplant patients

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**Effects of the glutamate carboxypeptidase II (*GCP2* 1561C>T) and reduced folate carrier (*RFC1* 80G>A) allelic variants on folate and total homocysteine levels in kidney transplant patients.**

*Background.* The effect of the glutamate carboxypeptidase II *GCP2* 1561C>T and the reduced folate carrier 1 *RFC1* 80G>A polymorphisms on folate and total homocysteine (tHcy) plasma levels of kidney transplant patients are unknown.

*Methods.* In a cross-sectional study of 730 kidney allograft recipients, *GCP2* 1561C>T, *RFC1* 80G>A, folate, and tHcy plasma levels were analyzed using linear regression models that allowed dependent covariates to follow a gamma distribution for univariate and multivariate analyses.

*Results.* The allele frequency for *GCP2* 1561C>T was 0.05, and 0.43 for *RFC1* 80G>A. Heterozygosity or homozygosity for *GCP2* 1561C>T was associated with higher folate plasma levels compared to patients without mutation ($P < 0.0001$), while *RFC1* 80G>A showed no influence. Multiple testing, also including *MTHFR* 677C>T and *MTHFR* 1298A>C, revealed no interaction between the different genotypes and the folate plasma concentration. Neither *GCP2* 1561C>T nor *RFC1* 80G>A showed an association with tHcy plasma levels.

*Conclusion.* We conclude that *GCP2* 1561C>T is associated with elevated folate levels. *GCP2* 1561C>T and *RFC1* 80G>A are no major determinants of tHcy plasma levels in kidney transplant patients.

Elevated plasma levels of total homocysteine (tHcy) are associated with several adverse outcomes such as cardiovascular disease or neural tube defects in the general population [1]. The relevance of tHcy as a risk factor is even more pronounced in patients with chronic kidney disease on renal replacement therapy due to an altered homocysteine metabolism in such patients. [2] Over the past decade, several contributions have shown that genetic polymorphisms might determine plasma tHcy levels, either directly, or by affecting plasma folate levels. Mutations of the 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) have been studied extensively in a variety of populations and found to be associated with low folate levels and elevated tHcy plasma concentrations [3]. More recently, two more polymorphisms have been discovered and hypothesized to influence tHcy plasma levels: a 80G>A exchange within the reduced folate carrier 1 gene (*RFC1*; HUGO gene symbol: *SLC19A1*; solute carrier family 19, member 1) [4] and a 1561C>T transition of the glutamate carboxypeptidase II gene (*GCP2*: HUGO gene symbol: *FOLH1*; folate hydrolase 1) [5]. The *GCP2* 1561C>T mutation (H475Y) is located in exon 13 at the putative catalytic domain of the enzyme and is associated with a 53% reduction of enzyme acitivity [5]. *RFC1* 80G>A (R27H) occurs in exon 2 [4]. The functional consequence of this polymorphism on the encoded protein is currently unknown.

The *RFC1* gene product consists of 591 amino acid residues with a predicted molecular mass of 65 kD and is termed human folate transporter (FOLT). Alternative titles and symbols are placental folate transporter 1; reduced folate carrier protein (RFC); and intestinal folate carrier (IFC-1), which belongs to the SLC19A family of transporters. The human FOLT is an integral membrane protein. Transfection experiments with human *RFC1* cDNAs revealed an increased uptake of 5-methyltetrahydrofolate [6] and 5-methyltetrahydrofolic acid [7], suggesting involvement of the *RFC1* gene product in internalization of folates into cells. In mice, a 58 kD protein has been detected in the brush-border membrane

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of the intestine, which mediates intestinal folate transport [8]. Furthermore, the folate transporter is possibly important for the development of embryos, providing the folate transport across the placenta [9].

The *GCP2* gene product consists of 750 amino acid residues with an apparent molecular weight of 100 kD and is termed folylpoly-γ-glutamate carboxypetidase (FGCP). Alternative titles and symbols are prostate-specific membrane antigen (PSM); *N*-acetylated-α-linked acidic dipeptidase (NAALADase); folate hydrolase 1 (FOLH) [10]. Folylpoly-γ-glutamate carboxypetidase is an exopeptidase that is anchored to the apical brush-border membrane and shows folate hydrolase and *N*-acetylated-α-linked acidic dipeptidase activity. Folylpoly-γ-glutamate carboxypetidase hydrolyses the terminal glutamate residues of dietary folylpoly-γ-glutamates before absorption. Thereafter, the monoglutamyl folate derivatives are transported through the membrane via the folate transporter. Therefore, folylpoly-γ-glutamate carboxypetidase possibly regulates the availability of dietary folates [11].

The effect of *GCP2* 1561C>T and *RFC1* 80G>A on tHcy and folate plasma level has only been studied in a limited way, and the results are conflicting [12]. It was the purpose of this study to describe the respective frequencies of the *GCP2* 1561C>T and the *RFC1* 80G>A allelic variants in a large cohort of patients with a functioning kidney transplant and to test the hypothesis whether these genetic characteristics were independently associated with folate or tHcy plasma levels. Furthermore, we sought to test interactions among the various allelic variants of the *MTHFR*, *GCP2* and *RFC1* genes on folate and tHcy plasma levels.

## METHODS

### Study population

To assess the association between folate and tHcy plasma levels, and *RFC1* and *GCP2* genotype status, we conducted a cross-sectional study of 733 kidney allograft recipients who received routine follow-up at our institution. The detailed rules for study inclusion have been published elsewhere [13]. Material for genetic analysis was unavailable for three individuals, which left a final study population of 730 patients. None of the patients received routine folic acid or vitamin B supplementation. Important patient characteristics are shown in Table 1. The Institutional Review Board at the University of Vienna gave study approval. All patients provided written informed consent according to the Declaration of Helsinki and the Austrian Law on Gene Technology.

**Table 1.** Characteristics of 730 kidney graft recipients

| Variable | Mean (SD) or % |
|---|---|
| Age *years* | 51.8 (±13.1) |
| Gender *female/male* | 292/438 |
| Body mass index $kg/m^2$ | 25.4 (±4.3) |
| Estimated glomerular filtration rate *mL/min* | 56.0 (±20.1) |
| Time since transplantation *years* | 5.0 (±4.1) |

### Biochemical methods

Fasting citrated blood was immediately placed on ice, and centrifuged at 2000 × *g* at 4°C (20 minutes) within 60 minutes. Plasma aliquots and 500 μL of citrated blood for isolation of DNA were snap-frozen and stored at −70°C. Plasma concentrations of tHcy (free plus protein-bound Hcy) [14] were determined by automated high-performance liquid chromatography (HPLC) with reversed-phase separation and fluorescence detection using tri-*n*-butylphosphine as a reducing agent. Hyperhomocysteinemia was defined as tHcy levels above 15 μmol/L [14]. Intra-assay variability was between 1.4% and 1.7% and interassay variability was between 1.5% and 1.9% for tHcy concentrations of 15.9 and 6.9 μmol/L, respectively. Folate and vitamin $B_{12}$ plasma levels were measured with a radioassay (SimulTRAC-SNB, ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). Folate deficiency was defined as a plasma concentration of less than 3.4 nmol/L, and vitamin $B_{12}$ deficiency was defined as a plasma concentration of less than 118 pmol/L, respectively. Interassay variability was 4% to 5% for folate measurements and 4% to 6% for vitamin $B_{12}$ levels. The creatinine clearance was calculated using the equation of Cockcroft and Gault [15].

*RFC1* 80G>A was analyzed by restriction fragment length polymorphism (RFLP) analysis of a 230 bp polymerase chain reaction (PCR) product that had been amplified with the primer pair RFC1 (5′-AGTGTCA CCTTCGTCCC-3′; nucleotide 51–70, GenBank accession number: U19720) and RFC2 (5′-TCCCGCGTGAAG TTCTTG-3′; nucleotide 263–280, GenBank accession number: U19720). In the presence of the G allele, cleavage with *Cfo* I produced three fragments of 125 bp, 37 bp, and 68 bp. The mutant A allele diminished a restriction site resulting in two fragments of 162 bp and 68 bp, respectively. The RFLP system for determination of *GCP2* 1561C>T included GCP1 (5′-GGTGAGAATG ATGGACTTTAC-3′; nucleotide 46235–46255, GenBank accession number: AF007544) and GCP2 (5′-CTTTG AGCTCAGTTTCACTG-3′; nucleotide 46713–46732, GenBank accession number: AF007544) producing a 498 bp fragment that remained uncleaved by *Acc* I in the presence of the C allele. The mutant T allele was cut by *Acc* I into two fragments of 218 bp and 280 bp. Identification of the 677C>T transition and of the 1298A>C transversion in *MTHFR* was performed by RFLP [16, 17].

### Statistical analyses

The SAS for Windows (release 8.2) statistical software was used for all analyses (SAS Institute, Inc., Cary, NC).

**Table 2.** Vitamin status and total homocysteine (tHcy) plasma levels of 730 kidney graft recipients

| | Median (25th/75th percentile) |
|---|---|
| Plasma vitamin $B_{12}$ *pmol/L* | 220 (170/308) |
| Plasma folate *nmol/L* | 13.0 (10.0/16.9) |
| Plasma tHcy *μmol/L* | 15.0 (11.9/19.8) |

**Table 3.** Plasma folate and total homocysteine (tHcy) levels of 730 kidney graft recipients according to *GCP2* genotypes (mean ± SD)

| | *GCP2* 1561C>T genotype | | |
|---|---|---|---|
| | CC | CT | TT |
| Number | 654 | 74 | 2 |
| Plasma folate *nmol/L* | 14.7 ± 13.2 | 15.6 ± 7.1 | 24.7 ± 12.8 |
| Plasma tHcy *μmol/L* | 17.0 ± 8.6 | 17.6 ± 10.6 | 13.5 ± 4.9 |

**Table 4.** Plasma folate and total homocysteine (tHcy) levels of 730 kidney graft recipients according to *RFC1* genotypes (mean ± SD)

| | *RFC1* 80G>A genotype | | |
|---|---|---|---|
| | GG | GA | AA |
| Number | 243 | 352 | 135 |
| Plasma folate *nmol/L* | 15.2 ± 16 | 14.8 ± 11.9 | 14.4 ± 6.8 |
| Plasma tHcy *μmol/L* | 17.7 ± 10.2 | 16.8 ± 8.3 | 16.7 ± 6.8 |

**Table 5.** Plasma folate and total homocysteine (tHcy) levels of 730 kidney graft recipients according to *MTHFR 677* genotypes (mean ± SD)

| | *MTHFR 677*C>T genotype | | |
|---|---|---|---|
| | CC | CT | TT |
| Number | 344 | 307 | 79 |
| Plasma folate *nmol/L* | 15.3 ± 6.3 | 15.2 ± 17.8 | 11.7 ± 8.6 |
| Plasma tHcy *μmol/L* | 15.8 ± 7.1 | 17.1 ± 7.9 | 22.5 ± 14.7 |

We described important patient characteristics using mean values and standard deviations for continuous variables and percentages for categorical variables. As the distribution of plasma tHcy and folate levels was found to be skewed to the right, and therefore nonnormally distributed, we used a special case of general linear models for all advanced analyses. The link function was chosen to be linear and spread was determined to follow a gamma distribution (in SAS: PROC GENMOD, LINK = IDENTITY, DIST = GAMMA). Such an approach provides unbiased estimates of effect for positively skewed data without the need to conduct data transformation (e.g., to a logarithmic scale) [18, 19]. We used such models both for univariate and multivariate analyses. One set of analyses used plasma level of folate as the dependent covariate, a second set modeled plasma tHcy concentration. Independent covariates included patient age (in decades), gender, time since kidney transplantation (<2 vs. ≥2 years), and categories of underlying kidney disease (diabetic nephropathy, glomerulonephritis, interstitial nephritis, polycystic kidney disease, miscellaneous other defined, and kidney disease not otherwise defined). We calculated each patient's body mass index (BMI), categorized in quintiles as the weight in kilograms divided by the squared height in meters and defined quintiles of the plasma levels of folate and vitamin $B_{12}$, respectively. For each patient, we estimated the creatinine clearance/glomerular filtration rate (GFR) using the Cockcroft-Gault approximation [15] and then staged renal function in accordance with the recent guidelines of the National Kidney Foundation (NKF) (>90, 60 to 90, 30 to 60, <30mL/min/1.73 $m^2$) [20]. We created dummy covariates for all allelic combinations of nucleotides 677 and 1298 of *MTHFR*, as well as for heterozygosity and homozygosity of *RFC1* 80G>A. Patients with any mutation for the *GCP2* gene at nucleotide 1561 were put into one single category because there were only two patients who were homozygous. All two-way interactions between these dummy covariates were then tested for significance. However, such an approach raises a multiple-testing problem, where some interactions will be found significant by chance alone. Therefore, we used the approach described by Bonferroni to adjust the appropriate significance threshold (α level) for multiple testing [21].

## RESULTS

### Patients, vitamin status, and genotypes

The characteristics of all study participants are indicated in Table 1. The mean age in the cohort was 51.8 years, 60% were male, and more than half of the patients were obese or overweight (i.e., BMI >25 $kg/m^2$). Table 2 depicts vitamin status and plasma tHcy levels of all 730 kidney graft recipients. Exactly half of the patients had elevated tHcy (>15 μmol/L), only two patients (0.3%) were below normal for plasma folate (<3.4 nmol/L), and 66 patients (9.0%) had vitamin $B_{12}$ levels below the normal threshold (<118 pmol/L).

The allele frequency of *GCP2* 1561C>T was 0.05; 74 patients (study population prevalence 9.8%) were heterozygous and two patients (0.3%) were homozygous for the mutation (Table 3). Because of the small number of homozygous patients, we combined those with the heterozygous patients for further analysis. The allele frequency of *RFC1* 80G>A was 0.43; 352 patients (48.2%) were heterozygous and 135 patients (18.5%) were homozygous for this variant (Table 4). The allele frequency of *MTHFR* 677C>T was 0.32; 307 patients (42.1%) were heterozygous and 79 patients (10.8%) were homozygous for this variant (Table 5).

**Table 6.** Estimated univariate and multivariate linear associations between genotypes and plasma folate (FA) levels

| | Univariate results | | | Multivariate results[a] | | |
|---|---|---|---|---|---|---|
| Covariate | Estimate Δ*FA in nmol/L* | 95% CI | *P* value | Estimate Δ*FA in nmol/L* | 95% CI | *P* value |
| *RFC1* genotypes | | | | | | |
| 80GG | — | (Referent) | — | — | (Referent) | — |
| 80GA | −0.46 | (−1.62; 0.71) | 0.443 | −0.32 | (−1.40; 0.76) | 0.560 |
| 80AA | −0.77 | (−2.24; 0.70) | 0.304 | −0.08 | (−1.42; 1.26) | 0.907 |
| *GCP2* genotypes | | | | | | |
| 1561CC | — | (Referent) | — | — | (Referent) | — |
| 1561CT or 1561TT | 1.09 | (−0.68; 2.87) | 0.226 | 2.01 | (0.36; 3.65) | 0.017 |
| *MTHFR* genotypes | | | | | | |
| 677CC/1298AA | — | (Referent) | — | — | (Referent) | — |
| 677CC/1298AC | −1.39 | (−3.24; 0.47) | 0.143 | −1.05 | (−2.80; 0.70) | 0.241 |
| 677CC/1298CC | −0.03 | (−2.40; 2.35) | 0.983 | −0.08 | (−2.32; 2.17) | 0.948 |
| 677CT/1298AA | −0.48 | (−2.43; 1.46) | 0.626 | −0.49 | (−2.32; 1.34) | 0.597 |
| 677CT/1298AC | −1.30 | (−3.22; 0.62) | 0.185 | −2.15 | (−3.93; −0.38) | 0.018 |
| 677TT/1298AA | −4.40 | (−6.38; −2.42) | <0.0001 | −5.19 | (−7.06; −3.32) | <0.0001 |

[a] All multivariate models simultaneously account for age, gender, body mass index, estimated glomerular filtration rate, plasma levels of vitamin $B_{12}$, time since transplantation, underlying kidney disease, and all genotypes of *MTHFR*, *RFC1*, and *GCP2*

### Effect of *GCP2* and *RFC1* genotypes on plasma folate concentrations

Mean plasma levels of folate stratified by *GCP2, RFC1,* and *MTHFR* 677 genotypes are shown in Tables 3, 4, and 5. We tested whether *GCP2* or *RFC1* were associated with folate plasma levels using general linear models. In univariate analyses, we did not find any associations between the allelic variants of the *RFC1* or *GCP2* genes and plasma folate levels (Table 6). However, in a multivariate model controlling for age, gender, renal function, BMI, underlying renal disease, time since transplantation, and indicators for allelic status of *MTHFR* 677C>T and 1298A>C, we found that individuals with the heterozygous or homozygous variants of the *GCP2* gene had a plasma folate concentration that was, on average, 2.01 nmol/L higher compared to patients with the *GCP2* wild-type sequence (95% CI 0.36 to 3.65; $P = 0.012$). Plasma folate levels among *RFC1* allelic variants did not differ in multivariate models (Table 6).

When testing for combined effects of various allelic variants of the *MTHFR, RFC1,* and *GCP2* genes on plasma folate (i.e., effect modification), we found two specific combinations to be of potential relevance. Patients who had any mutation of the *GCP2* gene and were heterozygous in either the *MTHFR* gene at nucleotide 677 ($N = 9$; effect estimate of the interaction term −5.08 nmol/L; 95% CI −9.64; −0.53; non-Bonferroni adjusted, $P = 0.03$) or the *MTHFR* nucleotide 1298 ($N = 21$; effect estimate of the interaction term −3.95 nmol/L; 95% CI −7.51; −0.39; non-Bonferroni adjusted, $P = 0.03$). These findings would indicate that the association found between nonhomozygosity in the *GCP2* gene and elevated folate was not present in patients who were heterozygous for either of the two polymorphisms of *MTHFR* under study. However, using Bonferroni's adjustment for multiple testing, we found that neither of the interactions remained significant [21].

### Effect of *GCP2* and *RFC1* genotypes on plasma tHcy concentrations

Mean tHcy plasma levels according to *GCP2, RFC1,* and *MTHFR* 677 genotypes are indicated in Tables 3, 4, and 5. When testing whether *GCP2* 1561C>T or *RFC1* 80G>A were associated with tHcy plasma levels in regression models, we did not detect any significant associations between any of the allelic variants and tHcy plasma levels both in univariate and in multivariate analyses (Table 7).

When testing for combined effects of various allelic variants of *MTHFR, RFC1,* and *GCP2* on tHcy, we again found two specific combinations to be significant before adjustment for multiple testing: patients who had any mutation of the *GCP2* gene and were compound-heterozygous for both *MTHFR* polymorphisms or homozygous for the *MTHFR* 1298A>C mutation. As before, neither of the interactions remained significant after Bonferroni adjustment [21].

## DISCUSSION

In this study of 730 kidney transplant recipients, we analyzed whether allelic variants of two recently discovered genes, *RFC1* and *GCP2*, were associated with plasma levels of folate and tHcy. This population is the largest thus far to be studied with regard to this specific research question. Using general linear models and accounting for the skewed distribution of the outcomes variables we did not find any associations between *RFC1* genotype and plasma folate or tHcy, respectively. These findings arose from multivariate models that accounted for important potential confounders and predictors of the outcomes.

Similarly, we could not detect an association between plasma tHcy levels and either hetero- or homozygosity of the *GCP2* 1561C>T mutation. However, plasma fo-

**Table 7.** Estimated univariate and multivariate linear associations between genotypes and plasma total homocysteine (tHcy) levels

| | Univariate results | | | Multivariate results[a] | | |
|---|---|---|---|---|---|---|
| Covariate | Estimate Δ*tHcy in μmol/L* | 95% CI | *P* value | Estimate Δ*tHcy in μmol/L* | 95% CI | *P* value |
| *RFC1* genotypes | | | | | | |
| 80GG | (1.0) | (Referent) | — | (1.0) | (Referent) | — |
| 80GA | −0.87 | (−2.04; 0.29) | 0.142 | 0.06 | (−0.77; 0.88) | 0.890 |
| 80AA | −0.93 | (−2.41; 0.56) | 0.221 | −0.33 | (−1.36; 0.69) | 0.523 |
| *GCP2* genotypes | | | | | | |
| 1561CC | (1.0) | (Referent) | — | (1.0) | (Referent) | — |
| 1561CT or 1561TT | 0.45 | (−1.26; 2.16) | 0.609 | 0.11 | (−1.08; 1.29) | 0.860 |
| *MTHFR* genotypes | | | | | | |
| 677CC/1298AA | (1.0) | (Referent) | — | (1.0) | (Referent) | — |
| 677CC/1298AC | 1.27 | (−0.27; 2.82) | 0.106 | 0.37 | (−0.75; 1.49) | 0.518 |
| 677CC/1298CC | 1.56 | (−0.43; 3.55) | 0.123 | 0.74 | (−0.73; 2.22) | 0.323 |
| 677CT/1298AA | 2.39 | (0.75; 4.04) | 0.004 | 0.91 | (−0.30; 2.12) | 0.142 |
| 677CT/1298AC | 2.10 | (0.46; 3.74) | 0.012 | 0.87 | (−0.33; 2.07) | 0.154 |
| 677TT/1298AA | 7.63 | (5.30; 9.97) | <0.0001 | 3.65 | (1.84; 5.45) | <0.0001 |

[a] All multivariate models simultaneously account for age, gender, body mass index, estimated glomerular filtration rate, plasma levels of folate, plasma levels of vitamin $B_{12}$, time since transplantation, underlying kidney disease, and all genotypes of *MTHFR*, *RFC1*, and *GCP2*

late was found to be elevated in patients with these allelic variants compared to individuals without mutation (effect estimate +2.01 nmol/L; 95% CI 0.36 to 3.65; $P = 0.017$).

Regarding the *RFC1* allelic variants, these findings are consistent with the existing literature. Födinger et al [22] showed no major effect of *RFC1* 80G>A on tHcy or folate concentrations in a cross-sectional group of 120 chronic dialysis patients. The *RFC1* 80G>A variant did not demonstrate any association with folate status or tHcy plasma concentration among 169 French healthy adults, either [4]. In this study, however, Chango et al [4] showed that the *RFC1* 80GG/*MTHFR* 677TT genotype was associated with moderately higher tHcy plasma concentrations as compared to 80GG/677CC or 80GG/677CT genotype patients. Subjects with 80AA/677CT genotype showed higher plasma folate levels as compared to 80GG/677CT patients. There was no other effect of the *RFC1* polymorphism on plasma or red blood cell folate levels as measured by a microbiologic assay.

Devlin et al [5] found low levels of serum folate and elevated homocysteine to be related to the 1561C>T polymorphism of *GCP2* (H475Y) in 75 healthy Caucasians. [5] This observation was explained by a diminished activity of folypoly-γ-glutamate carboxypeptidase in H475Y-transfected COS-7 cells. Thus, this study suggested that the presence of the mutated *GCP2* allele impaired the intestinal absorption of dietary folates, resulting in relatively low blood folate levels and consequent hyperhomocysteinemia. Our findings that the 1561C>T mutation of the *GCP2* gene was associated with higher plasma folate levels in multivariate analyses are at odds with the observations of Devlin et al [5], and in better congruence with a previous study at our institution that showed similar plasma folate levels, but somewhat higher red blood cell folate and lower tHcy plasma levels among dialysis patients carrying a mutated T allele [22]. Similar to the present results, there was no effect of *GCP2* variants on homocysteine metabolism of dialysis patients in a multivariate analysis after controlling for some of the most important modifiers of the tHcy plasma level [22]. Vargas-Martinez et al [23] studied the associations between *GCP2* variants, and folate status and tHcy levels. In participants from the Framingham Offspring Study, they found *GCP2* 1561C>T to be associated with elevated plasma folate levels, but without effect on tHcy levels. We confirm these findings in renal transplant patients.

We did not find any interactions on folate or tHcy plasma levels among the allelic variants under study after Bonferroni adjustment for multiple testing. However, the present example illustrates how failure to account for multiple comparisons may lead to erroneous conclusions, especially in genetic studies where multiple comparisons are often inevitable [21].

While not the centerpiece of the study, we confirmed that homozygosity for *MTHFR* 677C>T was the most important genetic correlate of decreased plasma folate (both by significance and effect size), while compound heterozygosity for both the *MTHFR* 677T and 1298C also seemed to be modestly associated with lower plasma folate levels (Table 6). Clearly, homozygosity for *MTHFR* 677C>T remained the only genetic variant described thus far to be independently associated with increased tHcy (Table 7).

The present study has certain limitations. Findings from a population of renal transplant patients may not be representative of other populations. The cross-sectional design makes it possible that time-related biases are operational, most importantly survival bias. Certain genotype combinations might affect eligibility for transplantation or patient survival so that such patients are less likely to be represented in the analysis. However, such

a bias would solely influence prevalence estimates of various genotypes, but not necessarily associations between allelic variants and folate or tHcy plasma levels. Furthermore, a previous study has failed to detect an association between *GCP2* 1561C>T and survival in a high-risk population [24]. We did not assess dietary intake of folate, which should not be an issue, because none of the patients received folic acid or multivitamin supplements and so dietary folate intake can be assumed to be within a relatively narrow range. Finally, we neither used a microbiologic assay for estimation of folate supply, nor was red blood cell folate measured.

## CONCLUSION

From a large population, we provide compelling evidence that the recently described *RFC1* 80G>A and *GCP2* 1561C>T allelic variants are not major determinants of plasma homocysteine levels in patients with a functioning kidney graft. Therefore, we confirm similar findings from a smaller study of 120 dialysis patients [22] and from a case-control study nested in the Framingham Offspring Study Cohort [23]. Further research in other populations is necessary to ensure generalizability of these findings.

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